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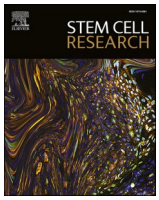

Lab Resource: Multiple Cell Lines

Characterization of two iPSC lines from patients with maternally inherited leigh (MILS) and neuropathy, ataxia, and retinitis pigmentosa (NARP) syndrome carrying the *MT-ATP6* m.8993 T>G mutation at different degrees of heteroplasmy

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A B S T R A C T

Human-derived experimental systems such as induced pluripotent stem cell (iPSC)-derived models are useful tools to study mechanisms and potential therapeutic approaches for mitochondrial disorders. Here, we generated two iPSC lines from fibroblasts of patients carrying mutations at *MT-ATP6* (m.8993 T>G). One patient with 96 % heteroplasmy suffered from Neuropathy, Ataxia, and Retinitis pigmentosa (NARP) syndrome, while the other patient with a homoplasmic mutation suffered from Maternally Inherited Leigh Syndrome (MILS). For reprogramming, we delivered reprogramming factors using Sendai virus and evaluated the pluripotency characteristics of the derived iPSCs. The degree of heteroplasmy remained stable after reprogramming.

1. Resource table

Unique stem cell lines identifier	*MDCi238-A* *MDCi239-A*
Alternative name(s) of stem cell lines	*Names commonly used by the researcher. none*
Institution	*Max Delbrueck Center (MDC)*
Contact information of distributor	*Markus Schuelke, MD, markus.schuelke@charite.de*
Type of cell lines	*iPSC*
Origin	*human*
Additional origin info required	*MDCi238-A (derived from fibroblast line 11692, female, 5 years)* *MDCi239-A (derived from fibroblast line 11398, female, 14 years)*
Cell Source	*Skin fibroblasts*
Clonality	*Clonal*
Method of reprogramming	*Episomal, using Sendai virus expressing* OCT3/4, SOX2, c-MYC, and KLF4
Genetic Modification	*Yes, Hereditary*
Type of Genetic Modification	*Wildtype cells derived iPSCs with a naturally occurring mtDNA mutation, no genetic modification done*

(*continued on next column*)

(*continued*)

Unique stem cell lines identifier	*MDCi238-A* *MDCi239-A*
Evidence of the reprogramming transgene loss	*PCR*
Associated disease	*Maternally Inherited Leigh Syndrome (MILS)* *Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP) syndrome*
Gene/locus	*Mutation m.8993 T>G in MT-ATP6*
Date archived/stock date	*MDCi238-A (25.03.2022)* *MDCi239-A (23.03.2022)*
Cell line repository/bank	*https://hpscreg.eu/cell-line/MDCi238-A* *https://hpscreg.eu/cell-line/MDCi239-A*
Ethical approval	*Ethical approval was obtained from the Institutional Review Board of Charité (Ethikkommission der Charité, Campus Virchow Klinikum) and registered under EA2/131/13*

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2. Resource utility

The ability to reprogram patient somatic cells into iPSCs followed by differentiation into specific lineages has become a useful tool for modeling mitochondrial diseases caused by mtDNA mutations (Galera-Monge et al., 2016; Henke et al., 2023; Lorenz et al., 2022). Such model systems can be used for metabolic analysis and drug discovery (Inak et al., 2017; Lorenz et al., 2017).

3. Resource details

Human ATP synthase is a multiprotein complex composed of 29 subunits, of which only two (MT-ATP6 and MT-ATP8) are encoded by the mitochondrial DNA (mtDNA). The MT-ATP6 subunit, together with an oligomeric ring of the c-subunit (c-ring), forms the channel through which protons cross the inner mitochondrial membrane. This proton flow is the driving force for the rotation of the c-ring and subsequent ATP synthesis. See (Table 1).

Several neuromuscular diseases have been linked to mutations in *MT-ATP6*. The most common mutation, m.8993 T>G, results in the replacement of a strictly conserved leucine residue by an arginine, p.(L156R). Mitochondrial mutations can be present either in all mtDNA molecules of a cell (homoplasmy), or only in a fraction of the mtDNA molecules (heteroplasmy).

For the generation of iPSC lines, we obtained skin fibroblasts from an adolescent female patient (fibroblast line 11398, 14 years) affected by NARP with 96 % heteroplasmy and from a female patient (fibroblast line 11692, 5 years) with a more severe phenotype (MILS) of a homoplasmic *MT-ATP6* mutation and introduced non-integrative Sendai viruses containing the reprogramming factors OCT3/4, SOX2, c-MYC, and KLF4 (Takahashi et al., 2007).

All lines expressed the characteristic morphology of embryonic stem cells, forming coherent and sharply delineated colonies (Fig. 1**A**, phase contrast images). Immunofluorescence revealed the presence of the pluripotency markers SOX2, OCT3/4, and the cell surface protein TRA-1–60 at passages 13–15 (Fig. 1**A**, epifluorescence images taken with a Leica DMi8 microscope). Using quantitative real-time PCR (Fig. 1**B**) and FACS (**Supplementary Fig. S1**), we examined the expression of the pluripotency marker genes *POU5F1*, *NANOG*, *DPPA4, SSEA4,* and *DNMT3B* and confirmed the absence of neuronal marker gene expression for *SOX1*, *PAX6*, and *NESTIN* at passages 13–15.

To determine the conservation of the *MT-ATP6* m.8993 T>G fibroblast heteroplasmy level in the derived iPSC lines, we used a quantitative PCR-restriction fragment length polymorphism (PCR-RFLP) method. The homoplasmic fibroblast line 11692 and the highly heteroplasmic fibroblast line 11398 (95.8 %) showed similar levels of heteroplasmy before and after reprogramming into iPSCs (97.2 % and 98.3 %, respectively, Fig. 1**C**). Both lines had maintained a normal karyotype at passages 4–5 (Fig. 1**D**).

An *in vitro* embryoid body (EB)-based evaluation (Fig. 1**E**) confirmed that both iPSC lines were able to differentiate into cell types of the three germ layers, as indicated by the ectodermal markers PAX6 and TUJ1, the mesodermal markers smooth muscle actin (SMA) and fibronectin (FN1), and the endodermal markers alpha-fetoprotein (AFP) and SOX17.

4. Materials and methods

4.1. Reprogramming

Patient-derived skin fibroblasts (11692 and 11398) were tested for the presence of HIV1/2 and Hepatitis B/C by a commercial diagnostic laboratory. We reprogrammed the parental lines using Sendai virus (CytoTune 2.0) according to manufacturer's guidelines. After reprogramming, the cells were PCR-tested for the absence of reprogramming vectors. For growth, iPSCs were cultivated in 6-well plate format using StemMACS iPS-Brew XF media (Miltenyi Biotec, 130–104-368) at 37°C

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography bright field	*Visual record of the line: normal*	Fig. 1 *panel A*
Phenotype	Qualitative analysis *(Immunocytochemistry)*	*Assess staining of pluripotency markers: OCT3/4, SOX2, Tra 1–60.*	Fig. 1 *panel A*
	Quantitative analysis *(RT-qPCR)*	*Assess mRNA rel. to GAPDH of pluripotency markers: OCT3/4, NANOG, SOX2, DPPA4, DNMT3B.*	Fig. 1 *panel B*
	Quantitative analysis *(Flow cytometry)*	Assess % of positive cells or transcripts for antigen & cell surface markers e.g. OCT3/4: 97.9 %, Tra 1–60: 88.2 %, NANOG: 98.7 %, SSEA-4: 98.6 %, SSEA-1: 0 %	***Supplementary file***
Genotype	*Karyotype and resolution*	*Virtual karyotyping using Illumina OMNI-EXPRESS-8v1.6 Chip. No significant changes compared to the primary cells detected. Co-amplification and three-color detection of nine human loci Identical to profile of primary cells*	Fig. 1 *panel D*
Identity	*Microsatellite PCR (mPCR) OR STR analysis*	*N/A*	
		Co-amplification and three-color detection of nine human loci Identical to profile of primary cells	*submitted in archive with journal*
Mutation analysis	Sequencing	*Heteroplasmy analysis by Sanger sequencing and RFLP*	Fig. 1 *panel C*
	Southern Blot OR WGS	*N/A*	
Microbiology and virology	Mycoplasma testing	*Mycoplasma testing by RT-qPCR. Negative*	***Supplementary file***
Differentiation potential	Embryoid body formation	*Immunostaining positive for SMA and fibronectin (mesoderm), AFP and SOX17 (endoderm), and PAX6 and TUJ1 (ectoderm)*	Fig. 1 *panel E*
Donor screening	HIV 1/2, Hepatitis B, Hepatitis C	*Negative*	*not shown but available from authors*
Genotype additional info	Blood group genotyping	*N/A*	
	HLA tissue typing	*N/A*	

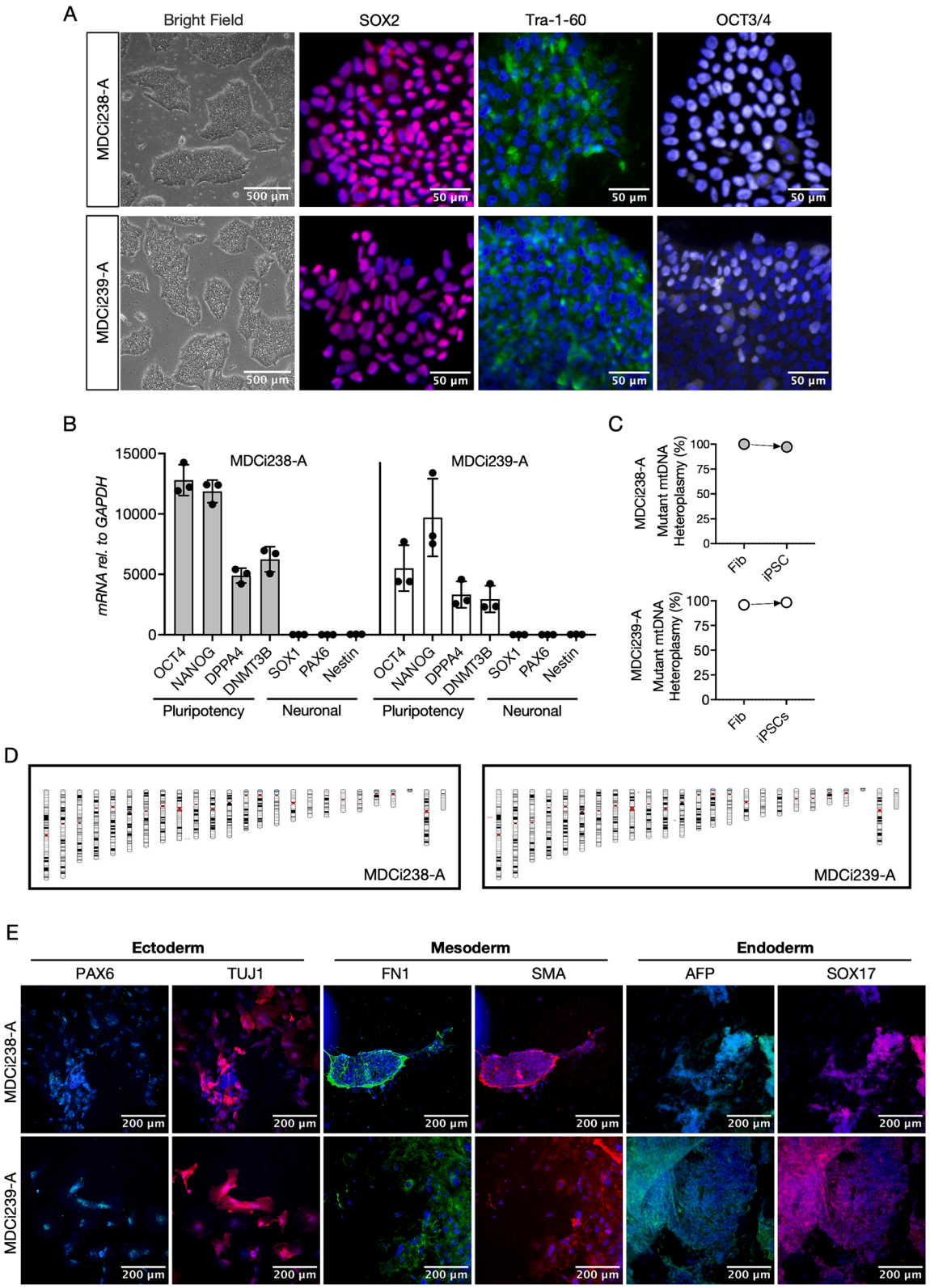

Fig. 1. Molecular and functional characterization of the iPSC lines. **(A)** Representative phase contrast images of the iPSC colonies and immunostaining of the pluripotency-associated markers SOX2, Tra-1-60 and OCT3/4 in the patient derived iPSCs. We counterstained the cells using DAPI; *scale bar*: 50 µm. **(B)** Quantitative RT-qPCR analysis of pluripotency-associated and neuronal markers in the iPSC lines. Data were normalized to the housekeeping gene *GAPDH* and are presented in triplicates (mean ± SD). **(C)** The heteroplasmic change during iPSC reprogramming. Levels of heteroplasmy of the source cells (fibroblasts) were maintained in the iPSCs. **(D)** Single nucleotide polymorphism (SNP)-based virtual karyotyping. We did not observe any larger areas of insertions or deletions; *green*: area with genomic gain; *red*: with genomic loss; *gray*: area with loss of heterozygosity. **(E)** Representative immunostainings of iPSCs differentiated *via* embryoid bodies (EBs) into cells belonging to the three germ layers: mesoderm (FN1, SMA), endoderm (AFP, SOX17), and ectoderm (PAX6, TUJ1); *scale bar*: 200 µm.

under hypoxic conditions of 5 % O_2, and 5 % CO_2.

4.2. Immunofluorescence staining for pluripotency markers

iPSCs were seeded onto GelTrex-coated (Gibco, A1413302) glass coverslips and cultivated at 37°C, 5 % O_2, and 5 % CO_2 until 60 % confluence. We fixed the cells with 4 % PFA in phosphate-buffered saline (PBS) solution for 15 min and then rinsed with PBS. For permeabilization, cells were incubated for 10 min with 0.1 % Triton X-100 (Merck, 108643) in PBS while rocking. After blocking with 10 % normal donkey/goat serum (abcam, ab7475/ab7481) in PBS for 1 h, we diluted the antibodies in 10 % serum and incubated overnight at 4°C. After three washing steps with PBS for 5 min, we added the corresponding secondary antibody in 10 % serum and incubated while shaking for 45 min at RT protected from light. For nuclear counterstain, we added 1:1,000 of DAPI (Invitrogen, D1306). The cells were washed three times with PBS and mounted on coverslips using Mowiol 4–88 (Roth, 0713). After immunostaining, cells were imaged with a Leica Thunder DMi8 microscope and further processed with the Leica Application Suite (LAS) X.

4.3. Formation of embryoid bodies (EBs) and differentiation of three germ layers

For the formation of embryonic bodies (EBs), we detached the iPSCs at about 80 % confluence using Dispase (ThermoFisher, 17105041). Cells were transferred into a 6-well non-tissue culture plate (Falcon, 351146) and cultured for seven days at 37 °C under hypoxic conditions in 5 % O_2, and 5 % CO_2 in StemMACS iPS-Brew XF media (Miltenyi Biotec, 130–104-368). We then transferred the EBs onto GelTrex-coated (GibCo, A1413302) glass coverslips and cultured them for 7 days for mesodermal and endodermal markers, and for 14 days for ectodermal markers at 37°C in 5 % CO_2 and 21 % O_2. EBs were stained as described above.

4.4. Quantitative real-time PCR (RT-qPCR)

We isolated total mRNA using the NucleoSpin RNA Plus XS kit (Macherey-Nagel, 740990) and reverse transcribed 1 µg RNA to cDNA using SuperScript IV reverse transcriptase (Invitrogen, 18090010) in 25

Table 2
Reagents details.

	Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency markers (IF staining)	anti-human OCT3/4 (C10), mouse	1:150	SantaCruz Biotechnology Cat #sc-5279	AB_628051
	anti-human SOX2, goat	1:200	R&D systems Cat #AF2018	AB_355110
	anti-human TRA1-60, mouse	1:200	Merck Cat #MAB4360	N/A
Pluripotency markers (FACS)	anti-SSEA4-VioBlue	1:50	Miltenyi Biotec: Cat #130–098-366	AB_2653521
	anti-OCT3/4-APC	1:50	Miltenyi Biotec: Cat #130–123-318	AB_2819472
	anti-TRA1-60-Vio488	1:50	Miltenyi Biotec: Cat #130–106-872	AB_2654228
	anti-SSEA1 (CD15) PE-Vio770	1:50	Miltenyi Biotec: Cat #130–114-012	N/A
	anti-NANOG-PE	1:50	Cell Signaling:Cat #14955S	N/A
Differentiation markers	anti-beta-Tubulin III (neuronal), mouse	1:600	SigmaAldrich: Cat #T8578	AB_1841228
	anti-PAX6, rabbit polyclonal	1:50	Invitrogen: Cat #42–1604	N/A
	anti-SMA, mouse	1:200	CellSignaling: Cat #48938S,	N/A
	anti-Fibronectin, rabbit	1:200	Abcam: Cat#ab299	AB_303474
	anti-AFP, rabbit	1:50	Proteintech: Cat #14550–1	N/A
	anti-SOX17, mouse	1:50	OriGene: Cat #CF500044	AB_2904161
Secondary antibodies	anti-mouse IgG Alexa Fluor 568, goat	1:200	Invitrogen: Cat #A11004	AB_2534072
	anti-rabbit IgG Alexa Fluor Plus 488, goat	1:200	Invitrogen: Cat #A32731	AB_2633280
	Primers			
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**	
Pluripotency markers (RT-qPCR)	*POU5F1 (OCT3/4)*	120 bp	FOR:GTGGAGGAAGCTGACAACAA, REV:ATTCTCCAGGTTGCCTCTCA	
	NANOG	78 bp	FOR:CCTGTGATTTGTGGGCCTG, REV:GACAGTCTCCGTGTGAGGCAT	
	SOX2	78 bp	FOR:GTATCAGGAGTTGTCAAGGCAGAG, REV:TCCTAGTCTTAAAGAGGCAGCAAAC	
	DNMT3B	93 bp	FOR:GCTCACAGGGCCCGATACTT, REV:GCAGTCCTGCAGCTCGAGTTTA	
	DPPA4	91 bp	FOR:TGGTGTCAGGTGGTGTGTGG, REV:CCAGGCTTGACCAGCATGAA	
Neuronal markers (RT-qPCR)	*NES*	79 bp	FOR:TTCCCTCAGCTTTCAGGAC, REV:GAGCAAAGATCCAAGACGC	
	PAX6	99 bp	FOR:GAATTCTGCAGACCCATGC, REV:TCTCGTAATACCTGCCCAG	
	SOX1	84 bp	FOR:TTGGCATCTAGGTCTTGGCTCA, REV:CGGGCGCACTAACTCAGCTT	
Housekeeping genes (RT-qPCR)	*GAPDH*	81 bp	FOR:CTGGTAAAGTGGATATTGTTGCCAT, REV:TGGAATCATATTGGAACATGTAAACC	
Mutation analysis	*mtDNA m.8993 T>G*	180 bp	FOR:AGCCTACTCATTCAACCAATAGCCC, REV:FAM-GGCGACAGCGATTTCTAGGA	
Sendai virus vectors	*SeV*	181 bp	FOR:GGATCACTAGGTGATATCGAGC, REV:ACCAGACAAGAGTTTAAGAGATATGTATC	
	SeV-Klf4	410 bp	FOR:TTCCTGCATGCCAGAGGAGCCC, REV:AATGTATGCAAGGTGCTC	
	SeV-cMyc	532 bp	FOR:TAACTGACTAGCAGGCTTGTCG, REV:TCCACATACAGTCCTGGATGATGATG	
	SeV-KOS	528 bp	FOR:ATGCACCGCTACGAGTGAGCGC, REV:ACCTTGACAATCCTGATGTGG	

µl. For quantification of pluripotency markers as well neuronal markers, 2 µl of the cDNA were evaluated by qPCR. Primer sequences have been described by Zink et al. 2021. All the expression values were normalized to the GAPDH housekeeping gene. Plots represent at least three independent experiments.

4.5. Karyotyping and STR-analysis

iPSCs were karyotyped using iSCAN by Illumina OMNI-EXPRESS-8v1.6 Chip (marker coverage 958,497 spanning the whole human genome). The analysis was performed by using Karyostudio 1.3 software based on the information of the GRCh36/hg18 dataset. STR analysis was performed by using the GenePrint® 10 System (Promega Corporation).

4.6. mtDNA heteroplasmy measurement

We determined heteroplasmy levels of the *MT-ATP6* m.8993 T>G mutation using PCR-restriction fragment length polymorphism (PCR-RFLP). iPSCs were harvested and genomic DNA was isolated using the Nucleo-Spin Tissue kit (Macherey-Nagel, 740952). DNA was then diluted to a concentration of 50 ng/µL. We generated FAM-labeled PCR-fragments with specific oligonucleotides (Table 2), which was cleaved by *Hpa*II (NEB, R0171) into 25 + 155 bp fragments only in the presence of the mutation. To evaluate the percentage of cleaved *versus* uncleaved fragments we used capillary electrophoresis and laser detection of the FAM-labeled RFLP-fragment using the 3500 Series Genetic Analyzer (Applied Biosystems, RRID:SCR_021901). Results were normalized to a standard curve.

4.7. Mycoplasma testing

We excluded contamination with *Mycoplasma* species using the qPCR-based Venor®GeM qOneStep Kit at cell lines passage 15–17 (Minerva Biolabs, 11–91025).

CRediT authorship contribution statement

Anna Maria Haschke: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sebastian Diecke:** Supervision, Resources, Methodology, Investigation. **Markus Schuelke:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103547.

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